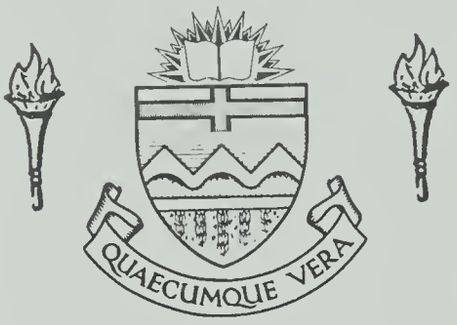

THE UNIVERSITY OF ALBERTA

A RAPID METHOD TO DETERMINE THE MAMMALIAN CELL CYCLE

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "A Rapid Method to Determine the Mammalian Cell Cycle" submitted by Gerald George Miller in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

A method is developed for the determination of the mammalian cell cycle and the duration of each of its major phases, mitosis, G_1, DNA synthetic period, and G_2. Mitosis is determined visually by assessment of the mitotic index at intervals after cells synchronized in mitosis are released from the mitotic block. The three remaining phases are ascertained from interpretation of a graphic representation of the uptake curve of tritiated thymidine by a synchronized population of cells grown directly in scintillation vials. The scintillation counting method for the determination of these parameters is advantageous over methods using autoradiography in that the investigator's bias in scoring cells is eliminated. Complex mathematical interpretations are unnecessary, and the data are readily obtained from the scintillation counter.

ACKNOWLEDGEMENTS

The advice of Dr. M.A. Russell concerning statistical methods of analysis is gratefully acknowledged. I am indebted to Dr. G.W.R. Walker for his continuing support and encouragement. This project was supported by funds from the National Research Council of Canada research grant to Dr. G.W.R. Walker.

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INTRODUCTION

Many problems in the biology of eukaryotic organisms are currently being further resolved through the use of synchronously growing mammalian cell cultures. The obvious advantage of studying populations of cells synchronized with respect to stage of the cell cycle lies in the fact that the cells are proceeding with the normal biochemical and biophysical events in unison (or almost so) and each event of interest may be singled out, simulating investigation of one individual cell. Synchronized cell cultures are presently yielding information regarding gene regulation, repair of DNA damaged by various external agents such as chemical mutagens and X rays, the mechanism of genetic recombination and many other notable events of cellular metabolism.

In using synchronized populations of cells it is usually necessary for the investigator to place the event of interest into one of the following four major phases of the cellular division cycle: mitosis (M), G_1, DNA synthesis (S), or G_2. A figure representing the cell cycle is included for reference.

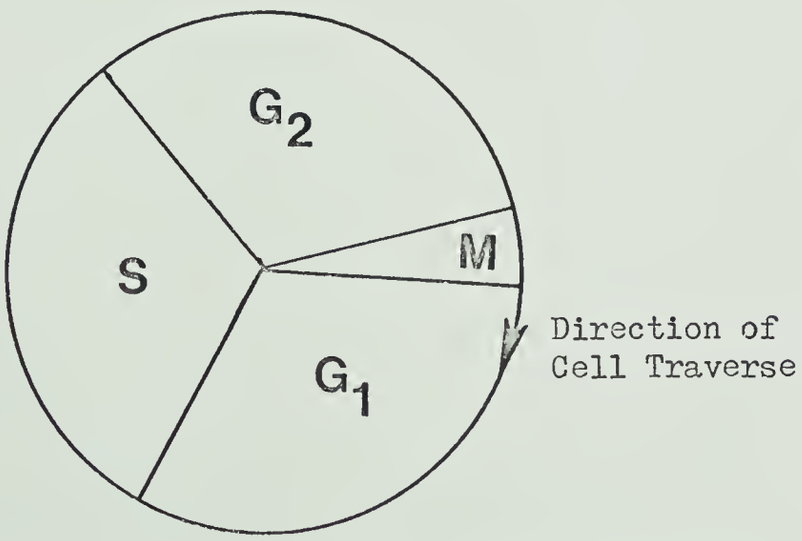


Fig. 1. Phases of the Mammalian Cell Cycle

The division cycle of a particular mammalian cell strain is typically variable in duration, depending upon such parameters as individual culture technique and history of the culture since the date of explantation. For this reason it is important that the investigator ascertain the duration of each phase of the cell cycle as it is expressed in his particular laboratory, rather than relying on figures obtained from the literature.

A technique for cell cycle determination is developed here which takes advantage of the specific uptake of tritiated thymidine into DNA in a predictable, stepwise manner. Cells are grown directly in scintillation vials and a scintillation spectrometer is used to trace uptake of labeled thymidine. The procedure is rapid and convenient, and yields the duration of each phase of the cycle. It is possible to determine the fraction of the cell population occupying each sector of the cycle through the use of equations derived by Puck, et al. (1963). The method is applied here to mammalian cells grown in monolayer culture, but could be adapted to cells propagated in suspension culture.

LITERATURE REVIEW

Since the discovery of Lajtha, Oliver and Ellis (1954), Lajtha, et al., (1955) that mammalian cells grown in vitro exhibited a life cycle that was divisible into four major phases, a number of techniques have evolved which were designed to characterize the duration of each phase, and the fraction of cells occupying each phase, of the cell cycle.

One of the earliest means of assessing the contributory stages of the cycle was used by Howard and Pelc (1953) and others (Lajtha, et al., 1958; Painter and Robertson, 1959; Dewey and Humphrey, 1962). This was the "pulse-chase" method which involved scoring for labeled vs. non-labeled mitotic cells harvested from an asynchronously growing culture which had been subjected to a brief pulse of tritiated thymidine. Use of this method is best confined to systems in which the mitotic index is high so that an inordinate number of cells need not be assessed (Cleaver, 1967). The same information may be derived from a "continuous labelling method" which has the advantage that the culture is not disturbed after addition of the tritiated thymidine (Cleaver, loc. cit.).

Dendy and Cleaver have combined autoradiography and microspectrophotometry (1964) to reveal the relative duration of the stages of cell progression and the fraction of the population occupying a given stage. The duration of the total cycle is not derived by this method, however, and the microspectrophotometry involved is laborious.

The most widely accepted method for cell cycle determination was devised by Puck and Steffen (1963). These investigators simultaneously added colchicine and tritiated thymidine to asynchronous

HeLa cell cultures and determined the cycle by counting labeled vs. non-labeled metaphase figures and the ratio of labeled to non-labeled cells in the culture. These data were applied to equations which enabled the authors to determine,

> a direct indication of randomness of any cell population; precise measurement of the duration of each recognizable division of the life cycle; the fraction of the cell population present at any time in various parts of the life cycle for random, singly phased, or polyphased cultures;. . .(Puck, loc. cit., p. 380.)

In light of the fact that each of the above methods requires lengthy microscopic analysis of the autoradiograms obtained, a method is proposed which eliminates autoradiography from the procedure. It is hoped that the advantage of the proposed method will lie both in increased accuracy through elimination of the investigator's bias in grain-counting, and in a more rapid estimation of the required data.

MATERIALS AND METHODS

Chinese Hamster Ovary cells, strain CCL-61 were obtained from the American Type Culture Collection Cell Repository, Rockville, Md. To reduce heteroploidy, the cells were cloned by selecting colonies derived from single cells plated at low dilution. To ensure purity, cloning was repeated, giving rise to a strain with 20 chromosomes. Cloning was performed as often as necessary to maintain the karyotype of the cell strain.

McCoy's 5a (modified) culture medium supplemented with 10% heat-inactivated calf serum, 5% heat-inactivated fetal calf serum, 50,000 IU/litre penicillin, 50,000 µg/litre streptomycin, 50,000 µg/litre neomycin, and 2.2 g/litre of sodium bicarbonate was used for monolayer culture of the cells. All tissue culture reagents were obtained from Grand Island Biological Company, Grand Island, N.Y. The cells were incubated in a humid atmosphere of 95% air and 5% CO_2 at 37°C.

During thymidine uptake experiments the cells were grown directly in scintillation vials (Packard Low Potassium-1 Vial, Packard Instrument Co., Inc., Downer's Grove, Ill.) containing 1.5 ml culture medium and 0.5 µc/ml of tritiated thymidine of specific activity 6.7 c/mmole (New England Nuclear, Boston, Mass.) Scintillation counting was done using a Picker Liquimat Liquid Scintillation Spectrometer, and the standard counting solution of scintillation grade toluene containing 4 g/litre PPO and 0.05 g/litre dimethyl POPOP (Packard Instrument Co., Inc., Downer's Grove, Ill.) was added to 0.05% by volume of Beckman Bio-solv 3 (Beckman Instrument Co., Fullerton, Ca.).

I. Determination of the Cell Cycle by Scintillation Spectrometry

A. Conditioning of Scintillation Vials

In order to promote cell attachment to the lower surface of the scintillation vials, it was found necessary to "condition" the vials by growing cells in them for several days. A 1.5 ml suspension of cells was added to each vial, which was then incubated for several days until the cells became stationary through exhaustion of the medium and overconfluency. The vials were washed in a mild detergent solution and rinsed with distilled water. The process was repeated at least once. Sterile conditions were not maintained until the vials were autoclaved prior to the actual experiment.

B. Cell Density Determination

The optimal cell population density was determined by inoculation of a series of vials with increasing densities of cells as determined with a hemacytometer (Hawksley Cristalite Counting Chamber, Lancing, Sussex). After a period of incubation of 30 hr, the vials were inspected, and the vials in which cells had not quite reached confluency were chosen as those which contained the optimal original cell density.

D. Cell Synchrony

Cells were synchronized in mitosis using the hydrodynamic shearing method of Robbins and Marcus (1964) as modified by Tobey, Anderson, and Petersen (1967). Loosened mitotic cells were harvested from monolayer cultures by 20-second shakes at intervals of 10 min. in a mechanical shaker. Cells collected in the first 7 shakes were discarded to improve synchrony, and cells collected over a period of 3 hr

were held in centrifuge bottles in an ice-water bath until collection by centrifugation at 2,000 rpm (4°C) for 15 min. (International PR-2 Refrigerated Centrifuge, head #284) Cells were resuspended to a final population density of 67,500/ml in 37°C medium containing 0.5 μc/ml tritiated thymidine. This was defined as T_0.

The degree of cell synchrony for each experiment was determined by scoring the mitotic index of the cell population at this time. Cells in metaphase, anaphase, and telophase were defined as mitotic. A 5 ml aliquot of cells was centrifuged at 800 rpm in an International Clinical Centrifuge and resuspended for 15 min in 1% sodium citrate hypotonic solution. The cells were collected from the hypotonic by a 5 min centrifugation at the same speed and resuspended in fixative (3:1 methanol:acetic acid). After one hour, the cells were resuspended in 0.3 ml of fixative and dropped onto a microscope slide which had been dipped in 70% methanol. The slide was ignited to spread the cells. A 1.5% solution of natural aceto-orcein was used to stain the nuclei, (15 min) followed by dehydration in a series of increasing concentrations of methanol-water solution. No coverslip was necessary. At least 500 cells were counted to determine the metaphase index.

E. Duration of Mitosis

Cells were synchronized in mitosis by the aforementioned method, collected and resuspended in 37°C medium. Aliquots of 5 ml of cell suspension were added to scintillation vials, and the cells were harvested at 15 min intervals to determine the metaphase index. The duration of metaphase was determined as the point at which a regression line determined from the points of separate experiments crossed the

abcissa in a plot of metaphase index vs. time.

F. Scintillation Counting

At T_0, a 1.5 ml aliquot of the suspension of synchronized cells was pipetted into four replicate scintillation vials corresponding to each hour of the experiment. The replicates were removed from the incubator at hourly intervals and the labeled medium was aspirated off. Two 5 ml washes of Hank's balanced salts solution were employed to remove exogenous label from the cells and vials. This solution was chilled ($4^{\circ}C$) to prevent further uptake of label by the cells. The vials were then placed in a freezer until it was convenient to proceed with scintillation counting.

At this time, 1.0 ml of Beckman Bio-solv 3 was added to each vial in order to lyse the cells and release the labeled DNA to the counting solution. After 1 hr, 19.0 ml of the previously described counting medium were added. The vials were given counts of 1 min in the liquid scintillation spectrometer.

G. Statistical Analysis

As an arbitrary means of assessing the tritiated thymidine uptake curve of the scintillation counting method, simple statistical methods were applied. Linear regression lines were drawn through series of points which were determined to belong to a specific phase or phases of the cell cycle as represented on the graphs. The mean ($\bar{X}$) and standard error ($SE_{\bar{X}}$) of the 4 replicate vials for each hour was determined in series. The cumulated mean $\pm$ 2 $SE_{\bar{X}}$ was compared with the mean of four replicate vials from the next hour determined separately ($\bar{X}_f$), and if the value of $\bar{X}_f$ fell within this range, the

point was included in the series. A series terminated when the point was rejected on these grounds. The linear regression lines were calculated on the basis of the relative count/min for all four replicates from each point in a series. (See Table IX., Appendix I.)

Within the S period there is a series of consecutive points which represent a time when thymidine uptake is at a maximum and all cells are in S. The slope of the regression line of these points is greater than that derived when all points determined to lie within S are used in calculation of the regression line. These "S_{max}" points were determined by choosing points which fell outside a 90% confidence interval of the regression line for the population of cells in S. (See Figure 7, Appendix I.) The period of maximal uptake was used to approximate the kinetics of a single cell traversing S, while the regression drawn from all points in S is more representative of the total cell population.

II. Autoradiographic Determination of the Cell Cycle

The cell cycle was determined according to the analysis of Puck and Steffen (1963). Several minor variations from the 1963 procedure are listed. A 0.05% solution of trypsin (Schwarz Bioresearch Inc., New York) in Hank's balanced salts solution was used to release the cells from the culture flasks. Plastic culture flasks (Becton, Dickinson and Co., Canada, Ltd., Clarkson, Ontario) were used during the exposure of the cells to labeled thymidine and colcemid. A 0.08 µg/ml solution of colcemid in Hank's balanced salts solution (Grand Island Biological Company, Grand Island, N.Y.) was used as the metaphase arrestor. Medium containing 0.05 µc/ml of tritiated thymidine was used to label nuclei. Slide preparations were stained

as previously mentioned before application of Kodak Liquid Emulsion NTB2 (Eastman Kodak, Rochester, N.Y.). The autoradiograms were exposed for 14 days at 4°C and developed in a Dektol-water (1:3) solution (Eastman Kodak) for 3 min. At least 500 cells were counted for each hour of the experiment. The autoradiographic cell cycle determination served as a control for the scintillation counting method.

RESULTS

A. Conditioning of Scintillation Vials

Two periods of incubation of cells in vials as described in Materials and Methods were usually found sufficient to allow maximum cell attachment and a cell morphology comparable to that observed when cells are cultured in standard glass prescription bottles.

B. Determination of Cell Density

A cell population density which would not alter the kinetics of the growth cycle was chosen, and is defined as that initial cell density which will not quite reach confluency before termination of the experiment. A series of replicate vials containing increasing densities of cells was inspected 30 hr after inoculation, and those vials which appeared almost confluent at that time indicated the desired initial cell density.

Table I. Determination of Optimal Cell Density

Initial Cell Density					
(Cells/ml)	300,000	67,500	16,875	4,218	1,056
(Cells/vial)	450,000	112,500	28,125	7,030	1,760
State After 30 hr	← Overconfluent	Optimum	Underconfluent →		

The autoradiographic cycle determination was done using 49,500 cells/ml of medium (245,000 cells/flask), a density which was approaching confluency after 20 hr. Duration of the experiment was 19 hr.

C. Uptake of Tritiated Thymidine by the Scintillation Vials

Vials containing 1.5 ml of unlabeled medium were incubated for 0 and 30 hr to determine the background radiation level. To ensure that the curve obtained was not an artifact of uptake of labeled

thymidine by the vials, several vials were injected with 1.5 ml of labeled medium. Replicate vials were washed in the previously described manner with Hank's balanced salts solution at times corresponding to the start and end of a cell cycle experiment.

Table II. Uptake of Label by Vials and Determination of Natural Background*

Contents of Vial	Count/min at 2 hr	Count/min at 30 hr
1.5 ml Unlabeled Medium	15	14
1.5 ml Labeled Medium	313	302

*Average of 2 replicate vials.

Natural background was deemed insignificant since experimental count/min ranged from approximately 300 to 180,000. The glass vials adsorbed ca. 309 count/min, but no increase in counts was observed after 30 hours' incubation. Since this figure was constant, it was not subtracted from raw count/min.

D. Cell Synchrony

An estimate of cell synchrony was made at T_0 for each experiment in order to ensure that the cell population would traverse a transition between phases of the cycle as rapidly as possible. The mitotic index of synchronized cultures is compared with the mitotic index of the T_0 stage in the autoradiography experiments, which represents an asynchronous cell population.

Table III. Estimate of Initial Cell Synchrony*

Experiment	Mitotic Index at T_0
Scintillation Method	
Experiment I.	0.796
Experiment II.	0.843
Autoradiography	
Experiment I.	0.039
Experiment II.	0.036

*More than 500 cells counted.

E. Duration of Mitosis

Counts of the mitotic index as it decreased from T_0 were made in order to establish the time of transition of mitosis to G_1.

Table IV. Duration of Mitosis*

Time	T_0	15 Min	30 Min	45 Min	60 Min
Experiment I.	0.796	0.683	0.355	0.038	0.000
Experiment II.	0.843	0.720	0.311	0.047	0.000

*More than 500 cells counted.

Regression lines drawn from the above data indicate that, for the degree of synchrony observed in these experiments, mitosis ends at 48 to 50 min. (See Figure 2.)

F. Analysis of Total Cell Cycle

Data from the scintillation counting experiments appear in Appendix I., and are summarized in Text Figures 3 (a.) and (b.). Data from the autoradiographic control experiments also appear in Appendix I. and are presented graphically in Text Figures 4 (a.) and (b.). Table V. summarizes the duration of the cycle and each of its phases as determined by both methods. For interpretation of the

thymidine uptake graphs for the scintillation counting method, see the Discussion.

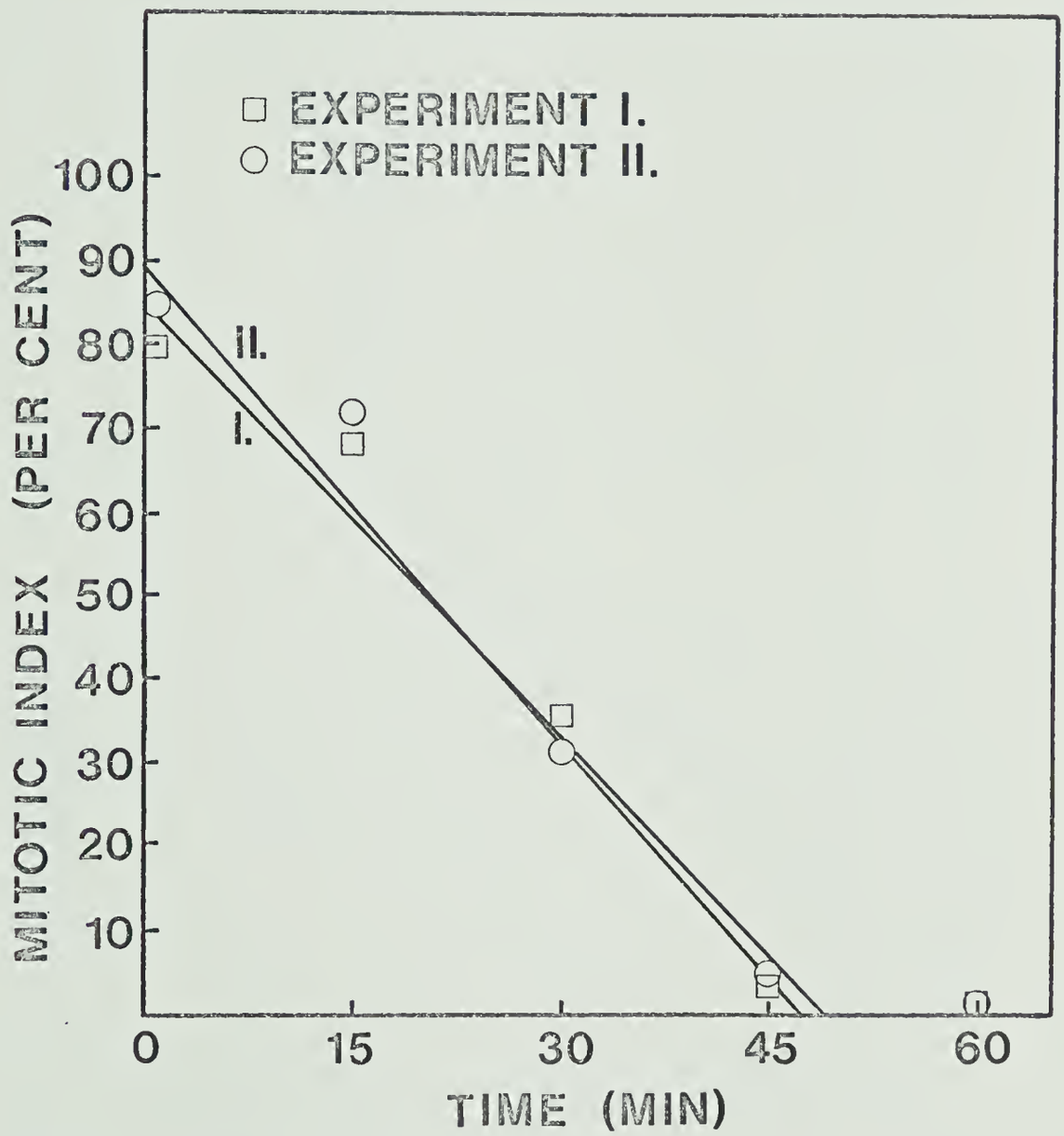


Fig. 2. Decline in Mitotic Index

I. and II. denote regression lines drawn from data of Experiments I. and II. Intersection of regression lines with abcissa represents the end of mitosis and beginning of G_1.

Figures III. (a.) and (b.) Uptake of Tritiated Thymidine by Chinese Hamster Ovary Cells

A Regression for first mitosis and G_1.

B Regression for first S period for single-cell kinetics.

C Regression for first S period for population kinetics.

D Regression for G_2, second M and G_1 periods.

E Regression for second S period.

Standard errors not shown too small to plot.

Duration of phases of cell cycle determined from intersections of regression lines as explained in the Discussion.

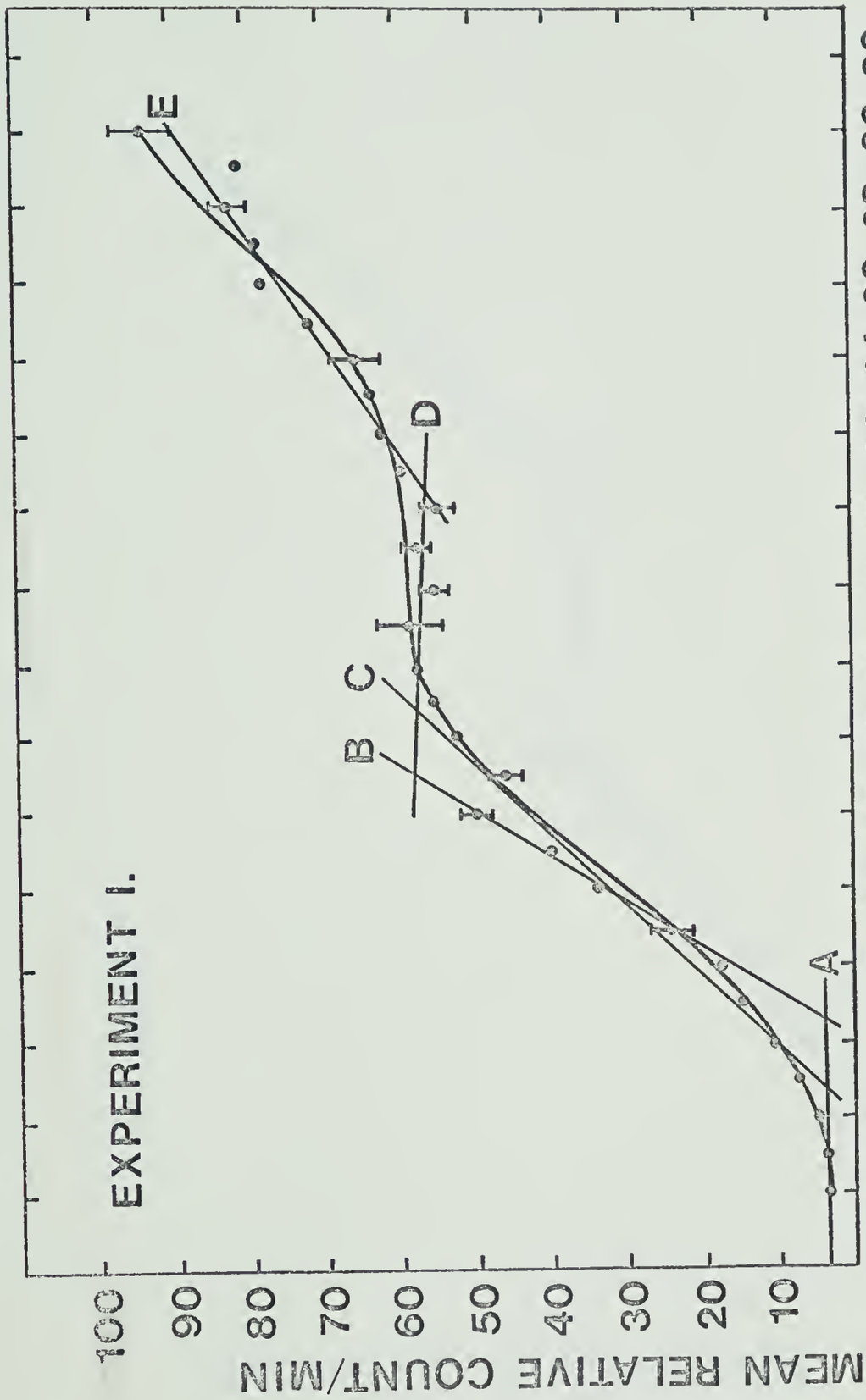


Fig. 3(a.) UPTAKE OF TRITIATED THYMIDINE BY CHINESE HAMSTER OVARY CELLS

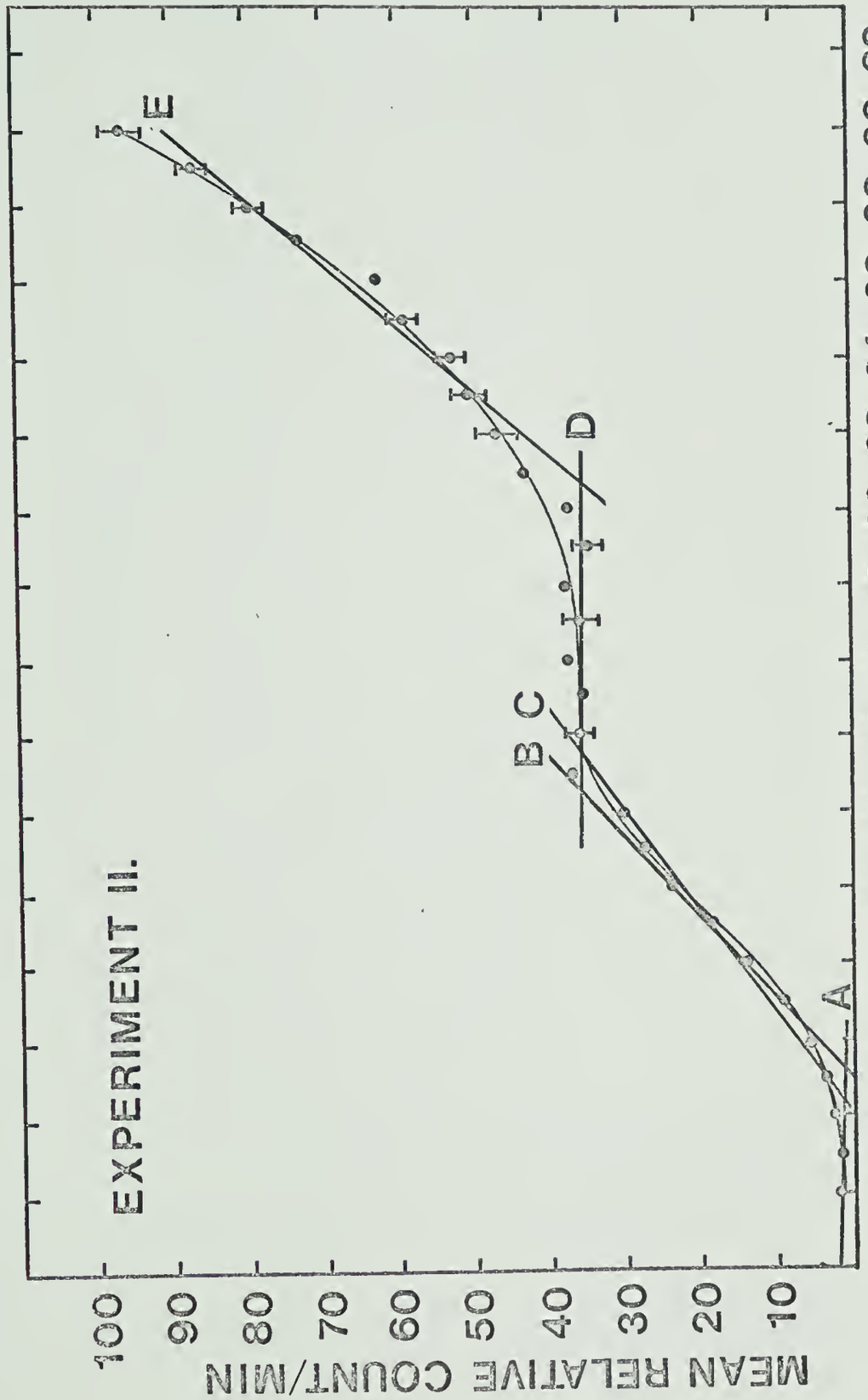
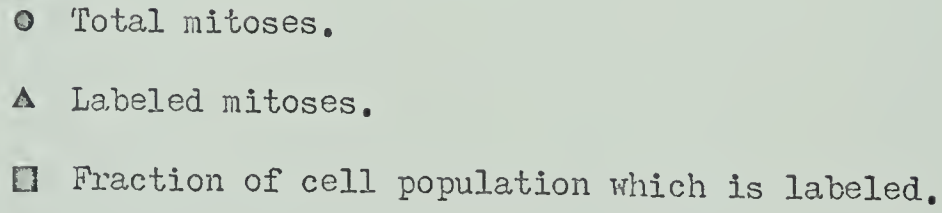


Fig. 3(b.) UPTAKE OF TRITIATED THYMIDINE BY CHINESE HAMSTER OVARY CELLS

Figures IV. (a.-d.) Determination of the Cell Cycle

Using Autoradiography



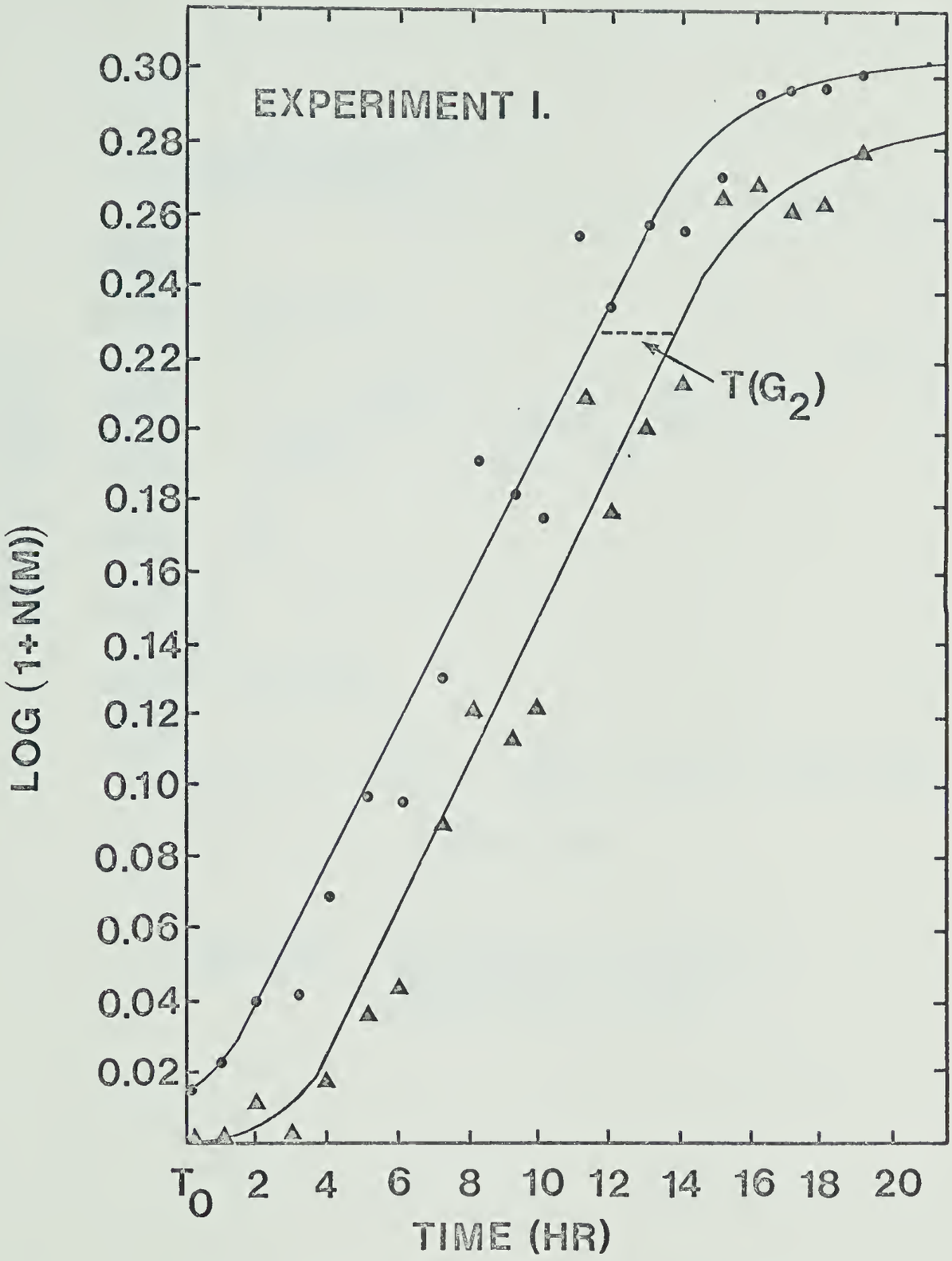


Fig. 4. (a.) ACCUMULATION OF MITOTIC CELLS

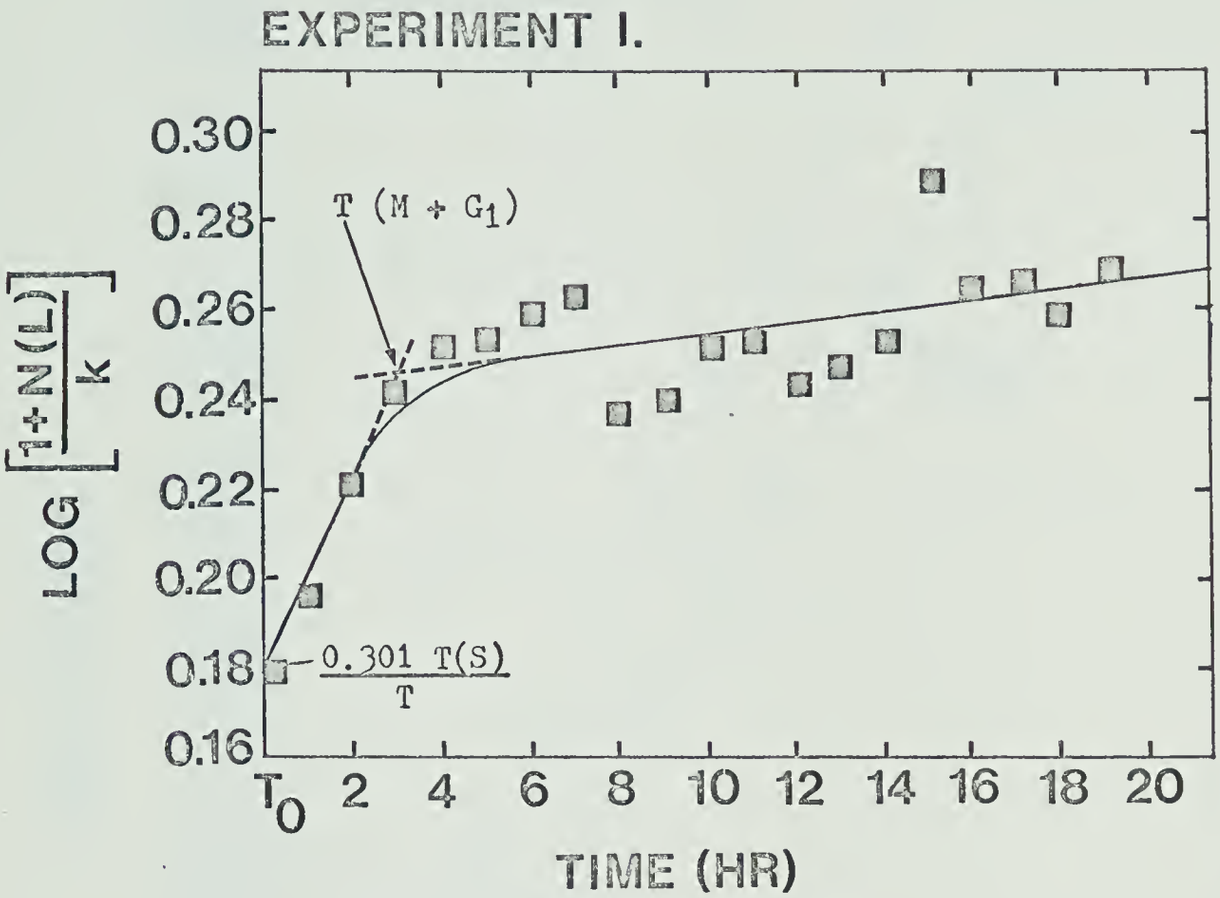


Fig. 4.(b.) ACCUMULATION OF LABELED CELLS

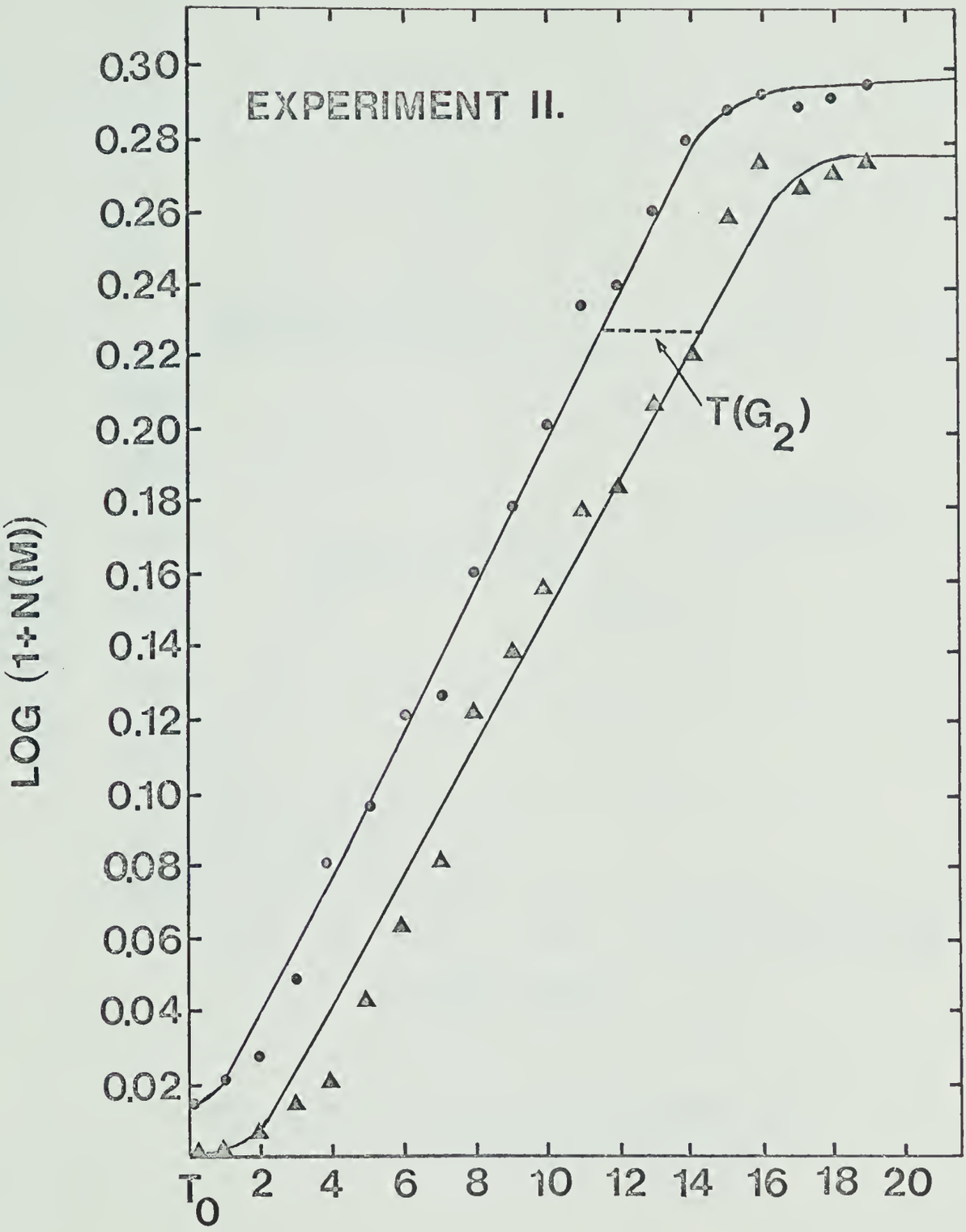


Fig. 4.(c:) ACCUMULATION OF MITOTIC CELLS

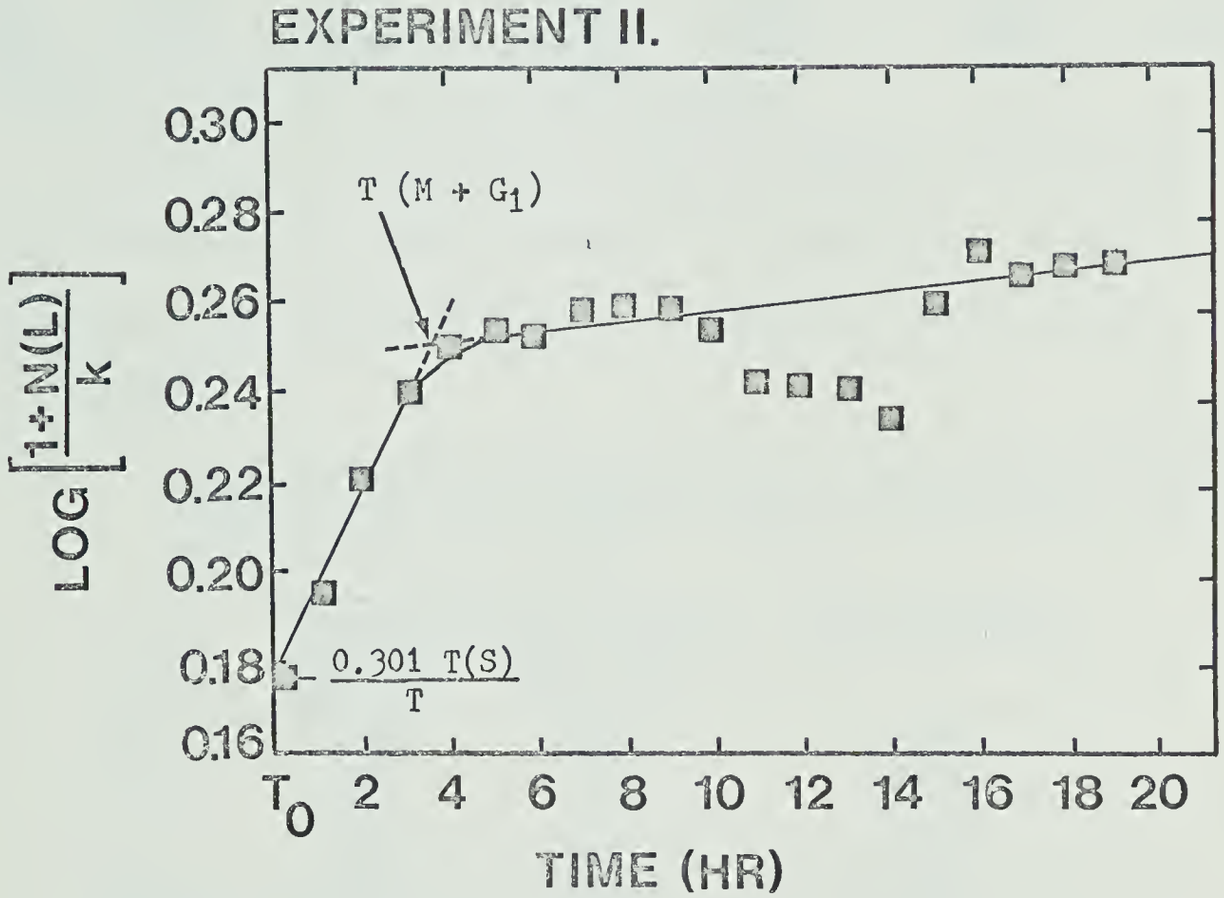


Fig. 4(d.) ACCUMULATION OF LABELED CELLS

Table V. Summary of the Data Derived From the Scintillation Counting and Autoradiographic Methods of Cell Cycle Determination. Duration of the Cycle and Each of Its Phases Given in Hours.

	Scintillation Counting				Autoradiography	
Phase	Population Kinetics		Single-Cell Kinetics			
	Expt. I.	Expt II.	Expt. I.	Expt. II.	Expt. I.	Expt. II.
M*	0.83	0.80	0.83	0.80	0.88	0.80
G_1	3.91	3.65	5.72	4.75	2.26	1.98
S	10.04	9.17	6.51	7.64	9.49	9.36
G_2	1.09	2.61	2.90	3.74	1.15	1.18
Total	15.87	16.23	15.87	16.23	15.91	15.79

*Prophase is included in G_2 in both methods.

For the sake of comparison of the two methods of cell cycle determination, it is useful to average the results from replicate experiments. The following table is a summary of the above data so averaged.

Table VI. Averaged Results of Experiments I. and II. for Scintillation Counting and Autoradiographic Cycle Determinations. Duration of Phases Given in Hours.

Phase	Scintillation Counting		Autoradiography
	Population Kinetics	Single-Cell Kinetics	
M	0.82	0.82	0.84
G_1	3.78	5.27	2.12
S	9.61	7.08	9.43
G_2	1.85	3.32	1.17
Total	16.05	16.05	15.85

A correlation of the data derived from the two methods is presented in the Discussion.

DISCUSSION

The distinct DNA synthetic period of eukaryote cells has been recognized for almost two decades (Howard and Pelc, 1953). Using as a model a population of cells which has been synchronized in mitosis, it is possible to predict the pattern of uptake of any DNA precursor which displays a reasonable degree of specificity. Thymidine is one such molecule and it has the added advantage of being readily available labeled with the hydrogen isotope, tritium. The following graph illustrates the theoretical uptake curve of tritiated thymidine by a population of eukaryote cells which have been synchronized in mitosis, as well as an interpretation of the phases of the life cycle: mitosis (M), G_1, DNA synthetic period (S), and G_2.

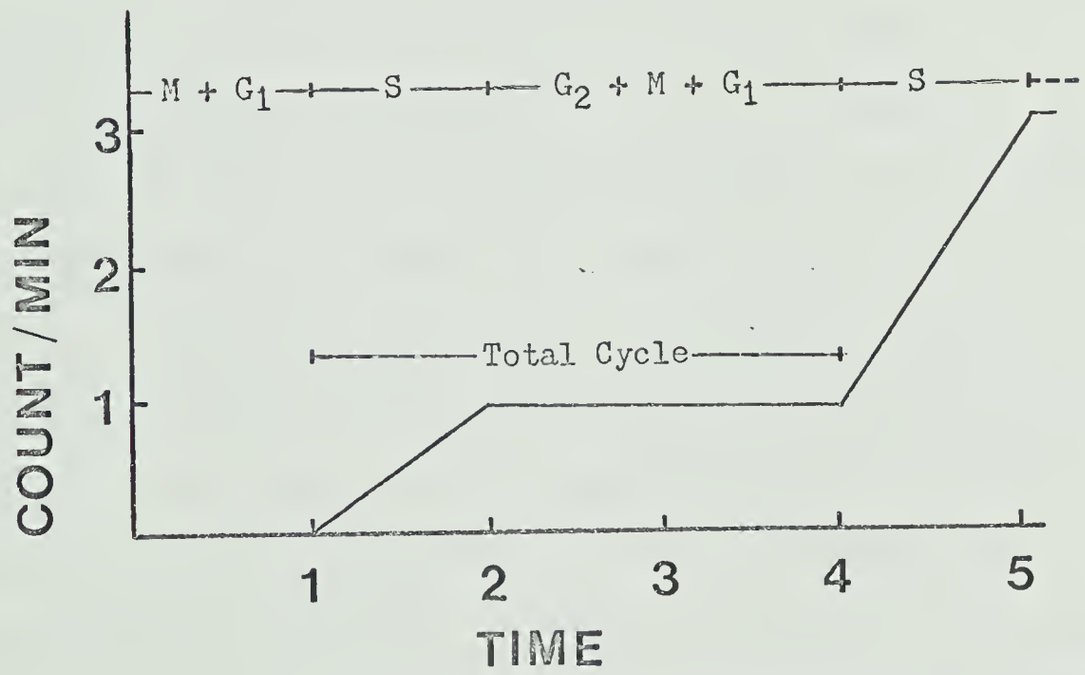


Fig. 5. Uptake of Tritiated Thymidine by Mammalian Cells Synchronized in Mitosis (Units on Axes Arbitrary)

Note that the slope for thymidine uptake in the second S period is greater than that of first S. This is due to the doubled cell number synthesizing DNA in the second S period, which lasts only as

long as the first.

The duration of mitosis plus G_1, or T $(M + G_1)$, is defined as that period, directly after release of the cells from the synchronizing agent, during which there is no increase in count/min. Duration of mitosis may be determined microscopically, and the length of G_1 is then ascertained by subtraction. The length of S is simply the duration of increase in count/min, and the total cycle is taken as the time difference between the beginning of the first S period and that of the second. Since there is no lag between phases of the cycle in exponentially growing cultures, G_2 is determined as the difference between total cycle and $(M + S + G_1)$ periods.

Certain differences in the thymidine uptake curve are noted in experimental situations. First, the initial horizontal $(M + G_1)$ plane is always seen to be elevated slightly from the abcissa. This is shown to be a result of adsorption of tritiated thymidine to the glass vials, but since this is constant throughout the experiment, it is not customary to subtract this background level from the count/min. Second, the uptake curve in experimental situations does not exhibit the sharp corners characteristic of phase transitions of the theoretical curve. Hence, regression lines are applied to the experimental curve to arbitrarily provide points on the graph delimiting phase transitions (see Figs. 3. (a.) and (b.)). The smooth curve results from asynchrony inherent in any cell population to which some method of synchronization has been applied.

As a result of the transient nature of cell synchrony, the slope of the curve for the second S phase is not quite as great as expected. For this reason, no measurements have been made past the

beginning of the second DNA synthetic period.

There are two means of assessing initial cell synchrony when using the scintillation counting method to determine the cell cycle of cultures which have been stopped in mitosis. The first is simply to ascertain the mitotic index before the mitotic block is released. In the case of mechanical selection followed by holding in chilled medium until enough cells are collected, it is possible to determine the fraction of cells which occupy a specific "window" of synchrony delimited by the interval of shaking collection. Little can be said for non-mitotic cells observed, except that it is likely they have progressed into early G_1. In this sort of collection of mitotic cells for synchrony, some of the non-mitotic cells could be dead or in a stationary phase. These cells would not pose a threat to synchrony as they are not likely to incorporate thymidine. Dead cells would be washed out of the vials with the labeled medium.

A second method of synchrony determination is to make use of a time during S when all cells theoretically should be taking up thymidine. A regression line drawn through several consecutive points in this region will have a sharper slope than a regression through all points which have been determined to lie within the S period (see Materials and Methods, and Fig. 7., Appendix I.). If the time difference between population and single-cell kinetics is significantly greater at the G_2 end of the S regressions than at the G_1 end, the factor of increasing asynchrony is readily determined. The factor of increasing asynchrony would be equal to the difference in time between population and single-cell kinetics taken at both ends of S, divided by T(S) for the cell population.

Several parameters will wield influence on the shape of individual experimental curves obtained using the scintillation method. Within reasonable bounds, these are found not to greatly influence the times of the cell cycle as derived from the curves. Degree of cell synchrony has been mentioned as a factor which influences the shape of the curve. Total asynchrony would give only a general upward trend in measured uptake of tritiated thymidine. Complete synchrony should give a curve more approaching the theoretical one.

Initial cell density and concentration of tritiated thymidine are interrelated parameters which may alter the shape of the curve. Too few cells in the vials can cause an apparent increase in duration of the total cycle, since in many strains of mammalian cells in vitro it is found that a certain minimal dilution of cells is required to promote growth and survival (Eagle and Piez, 1962.). Similarly, too high an initial cell population density may result in premature attainment of lag phase. For these reasons the optimal density of cells was determined as that initial inoculation density which would prevent the cells from quite reaching confluency by the end of the experiment. Also, presence of too many cells will flatten the slope of the second S phase curve while increasing that of the first, by drastically altering the concentration of exogenous thymidine in the medium. In statistical considerations, the highest number of cells possible is advantageous, as the variance in count/min is reduced as cell population density is increased and thymidine uptake is maximized. Standardized initial cell density and tritiated thymidine concentration help to negate minor variations in the appearance of the thymidine uptake curve.

In any biological experiment, the use of radioisotopes is to be avoided, if possible. The autoradiographic determination required 0.05 μc tritiated thymidine/ml of medium, while 0.5 μc tritiated thymidine/ml was required for the scintillation counting method. Although 0.5 μc/ml is not considered toxic to cells over a 30-hr period by some investigators, (Burki and Okada, 1969.) it is best to use as little of any radioisotope as is possible. Tritium is a very weak beta-emitter with a path length in solids of less than 2μ. As opposed to radioisotopes of higher energy (and longer path length) its biological damage is concentrated to one small volume. As tritiated thymidine is concentrated preferentially in the DNA molecule, the greatest damage to the cell will occur in its genetic material. If there is a serious radiological effect on the DNA, it will be expressed as a lag in the duration of the cell cycle (Sinclair, 1968., Varga and Várterész, 1969.). To test this possibility, the average of the slopes for the increasing proportions of mitotic cells in the population from the autoradiography experiments was compared with a theoretical slope calculated from the averaged duration of the cell cycle as determined by the scintillation counting method. (See Figures 4. (a.) and (c.) and Appendix I.) The means of comparison was Student's t-test, and the difference was not found to be significant.

One unexpected feature was noted in both experimental thymidine uptake curves. At 20 hr in Experiment I., and 19 hr in Experiment II., there was a significant decrease in count/min (see Figures 3. (a.) and (b.)). The nature of this depression is not known, but it is noted that both are points late in the second G_1 period. A

decrease in count/min is explicable only on grounds of turnover of the nucleotide bases in DNA during G_1. This is not an unreasonable supposition if one considers an excision-repair mechanism operable in the restoration of DNA damaged by the incorporated tritium. According to Smets (1969.) the mechanism of repair of radiation damage which is dependent on uptake of labeled DNA precursors is essentially terminated 4 hr after the damage has occurred. The points which display decreased count/min are beyond 4 hr from the last major uptake of tritiated thymidine. Perhaps a second repair mechanism is operable which restores the original DNA constitution by excision and subsequent repair of the lesion with bases derived from a "salvage" (unlabeled) pathway. Displacement of loosened cells from the vials during G_1 would not seem to be the reason for the depression in count/min since it is possible to rinse excess tritiated thymidine out of the vials during mitosis without apparent cell loss.

Firm cell attachment is a major requisite of the scintillation method. New scintillation vials do not support optimal attachment of Chinese Hamster Ovary cells and must be conditioned by growing cells for approximately one week in monolayer cultures. The factors involved in conditioning the vials are unknown, but include such parameters as removal of ions from the glass which would be deleterious to cell growth and attachment, alteration of the electrical charge on the vial surface by cellular deposits, etc. It is probable that any cell line which can be adapted to grow in glass culture bottles would readily lend itself to cell cycle determination by the scintillation method.

It is now thought that colcemid can alter the pattern of DNA synthesis and affect cell cycle kinetics (Fitzgerald and Brehaut, 1970.). Its use in cell cycle determinations therefore becomes questionable. The method developed here does not depend on the use of colcemid. The mechanical collection of mitoses has the advantage that all cells have had to traverse the final 10 min of G_2, and therefore may not unreasonably be assumed to be metabolically normal (Petersen et al., 1970.). Indeed, the scintillation counting method is amenable to cells synchronized at any point in the cycle and is thus useful in many experimental situations where data on cell cycle kinetics are necessary.

It is most reasonable to compare data based on population kinetics as derived by the scintillation counting method with the population-oriented autoradiographic data. At 0.82 hr and 0.84 hr, the figures derived for the duration of mitosis agree well. Data for G_1, however do not so closely agree. The scintillation counting method gives $T(G_1)$ as 3.78 hr, which is in closer agreement with 4.7 hr as determined by Puck, Saunders and Petersen (1964) than is the 2.12 hr determined by autoradiography. It is felt that the autoradiographic determination of G_1 is the least reliable of the methods, since calculation of G_1 has many variables when compared with the relatively direct scintillation method. (See Appendix I. for calculation of G_1 from autoradiographic data.) The duration of S phase as determined by both methods is in reasonable agreement at 9.61 hr for the scintillation method and 9.43 hr for the autoradiography. The G_2 periods agree reasonably well at 1.85 hr, and 1.17 hr for the autoradiography. Here, again, the number of variables

involved in the determination is less for the scintillation counting method.

Analysis of variance between the two methods as an indication of the relative accuracy of the scintillation counting method as based on two experiments would not be very meaningful (Russell, personal communication). It is, however, possible to discuss the accuracy of the method in general terms. The following figure is reproduced from data of Experiment II. The dark areas represent the intersection of 90% confidence intervals plotted about the first and second S--period regressions with that plotted about the G_2-M-G_1 regression.

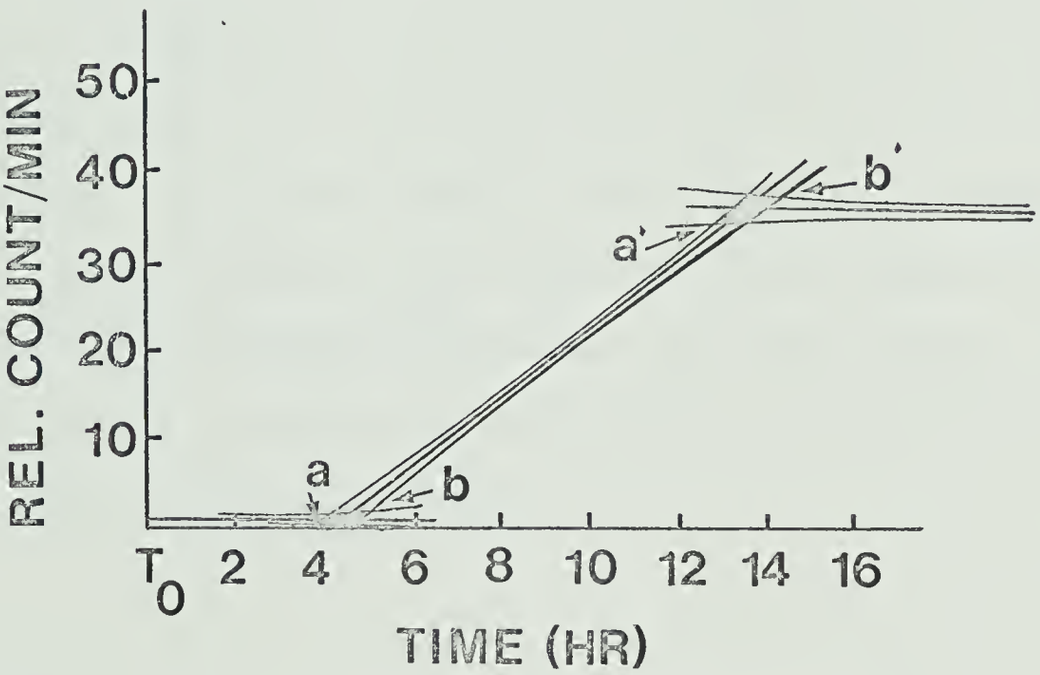


Fig. 6. Accuracy of the Scintillation Counting Method.

The points a and b' represent the outside estimate of S period if the regressions intersected there. For a 90% confidence interval, the probability that the regressions willintersect at a is 1/10 x 1/10, a very low probability. Through similar reasoning, the probability of the regressions traversing b' is 1/100. If the regressions were to intersect at both a and b', the overestimate of S would be 1.2 hr.

This probability is equal to 1/100 x 1/100, and can be considered negligible. In other words, the estimate of S would be as far away as 13% only one in ten thousand times. Similar reasoning prevails for an underestimate of S, if the regressions were to traverse points b and a'.

Since synchronized cell populations may behave slightly differently than do asynchronous ones, methodological consistency is desirable. An investigator working with synchronized cells should determine the cycle using synchronized populations, in order to stay within the frame of the model with which he is working.

The data derived from scintillation counting cell cycle experiments can be applied to the equations of Puck and Steffen (1963) to determine the fraction of the population which is occupying each cycle phase. Techniques involved in the experiment are readily carried out by technical staff, where this is not possible for the analysis of autoradiographic data. It is felt that the method developed here is valid as one of the methods of determination of the cell cycle and each of its phases.

SUMMARY

A method of analysis of the eukaryote cell cycle has been developed which, through its adaptability, closely approximates many experimental situations. The scintillation counting method is relatively simple and convenient when compared with autoradiographic methods. High accuracy is attainable through elimination of the investigator's bias in determination of all phases except mitosis, which is determined in a conventional manner. Complex mathematical analysis is eliminated, and the results are obtainable quickly through the analysis of two simple graphs.

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APPENDIX I.

The following tables present data from the scintillation counting and autoradiographic cell cycle determinations. Data from the scintillation experiment are converted to a relative scale based on the vial in each experiment which showed the greatest number of count/min.

Table VII. Uptake of Tritiated Thymidine by Chinese Hamster Ovary Cells

Time (hr)	Mean Raw Count/min		Mean Relative Count/min		Std Error of Mean Relative Count/min	
Expt.	I.	II.	I.	II.	I.	II.
2	5,895	787	3.34	1.14	0.42	0.10
3	6,210	891	3.52	1.29	0.10	0.15
4	8,286	1,511	4.70	2.18	0.06	0.17
5	12,472	2,584	7.07	3.74	0.66	0.30
6	18,206	4,120	10.32	5.96	0.28	0.44
7	26,073	6,548	14.79	9.47	0.28	1.22
8	30,475	9,673	17.28	13.99	1.32	0.56
9	41,429	13,053	23.49	18.87	2.86	0.15
10	59,637	16,763	33.82	24.24	1.74	0.90
11	69,818	18,750	39.59	27.11	1.61	1.17
12	85,857	20,536	50.56	29.93	2.06	0.68
13	80,879	35,593	45.86	37.00	2.49	1.31
14	92,616	25,104	52.52	36.30	0.88	1.88
15	97,742	24,857	55.43	35.94	0.78	1.17
16	101,873	25,801	57.77	37.30	1.48	1.09
17	103,808	24,896	58.87	36.00	4.43	2.63
18	96,991	26,256	55.00	37.96	2.04	0.97
19	101,947	23,633	57.81	34.17	2.10	2.11
20	96,400	25,853	54.67	37.38	2.20	1.36
21	105,668	29,489	59.92	42.64	1.51	0.54
22	109,200	32,324	61.92	46.74	1.31	2.83
23	112,662	34,609	63.89	50.04	1.53	2.44
24	115,901	36,221	65.72	52.37	3.29	2.08
25	126,050	40,421	71.48	58.44	1.07	1.91
26	138,304	42,945	78.43	62.09	1.70	1.96
27	138,266	50,156	78.41	72.52	1.99	0.55
28	145,015	55,095	82.23	79.66	2.58	2.83
29	143,338	60,794	81.28	87.90	1.29	2.14
30	165,036	66,504	93.59	96.16	4.05	2.80

Tables VIII. (a.) and (b.) Frequencies and Types of Cells in Asynchronous Populations of Chinese Hamster Ovary Cells Labeled with Tritiated Thymidine

* denotes labeled metaphase cells.

N(L) denotes fraction of cell population which is labeled.

$\frac{T(G_2)}{T}$

k = 2 (see Appendix I.)

Table VIII. (a.) Experiment I. Frequency of Various Cell Types in an Asynchronous Culture of Chinese Hamster Cells Labeled with Tritiated Thymidine

Time (hr)	Unlabeled Mitoses, N(U)	Labeled Mitoses, N(M*)	Logarithm 1 + N(M*)	Total Mitoses N(M)	Logarithm 1 + N(M)	Labeled Interphase	Total Label	Logarithm $\frac{1 + N(L)}{k}$
T_0	0.039	0.000	0.0000	0.039	0.0165	0.535	0.535	0.1786
1	0.041	0.009	0.0038	0.050	0.0212	0.590	0.599	0.1960
2	0.065	0.030	0.0128	0.095	0.0394	0.665	0.695	0.2203
3	0.081	0.019	0.0081	0.100	0.0414	0.762	0.781	0.2410
4	0.127	0.040	0.0170	0.167	0.0671	0.783	0.823	0.2510
5	0.165	0.086	0.0358	0.251	0.0973	0.748	0.834	0.2536
6	0.139	0.104	0.0430	0.243	0.0945	0.755	0.859	0.2593
7	0.120	0.227	0.0889	0.347	0.1294	0.652	0.879	0.2639
8	0.231	0.319	0.1203	0.550	0.1903	0.444	0.763	0.2367
9	0.220	0.297	0.1130	0.517	0.1810	0.478	0.775	0.2397
10	0.175	0.321	0.1209	0.496	0.1750	0.503	0.824	0.2511
11	0.160	0.632	0.2127	0.801	0.2555	0.198	0.830	0.2526
12	0.216	0.500	0.1761	0.716	0.2345	0.283	0.783	0.2415
13	0.190	0.617	0.2086	0.807	0.2569	0.192	0.809	0.2477
14	0.169	0.633	0.2130	0.802	0.2558	0.197	0.830	0.2526
15	0.007	0.860	0.2695	0.867	0.2711	0.133	0.993	0.2887
16	0.110	0.858	0.2691	0.968	0.2941	0.030	0.888	0.2657
17	0.147	0.824	0.2610	0.971	0.2947	0.028	0.852	0.2677
18	0.143	0.832	0.2630	0.975	0.2956	0.024	0.856	0.2586
19	0.098	0.897	0.2781	0.995	0.3000	0.004	0.901	0.2688

Table VIII.(b.) Experiment II. Frequency of Various Cell Types in an Asynchronous Culture of Chinese Hamster Cells Labeled with Tritiated Thymidine

Time (hr)	Unlabeled Mitoses, N(U)	Labeled Mitoses, N(M*)	Logarithm 1 + N(M*)	Total Mitoses N(M)	Logarithm 1 + N(M)	Labeled Interphase	Total Label	Logarithm $\frac{1 + N(L)}{k}$
T_0	0.036	0.000	0.0000	0.036	0.0153	0.530	0.529	0.1769
1	0.038	0.010	0.0043	0.050	0.0212	0.587	0.597	0.1951
2	0.047	0.018	0.0077	0.065	0.0273	0.681	0.699	0.2217
3	0.085	0.033	0.0141	0.118	0.0484	0.751	0.784	0.2417
4	0.156	0.048	0.0204	0.204	0.0806	0.775	0.823	0.2509
5	0.146	0.104	0.0430	0.250	0.0969	0.732	0.836	0.2539
6	0.167	0.157	0.0633	0.324	0.1219	0.676	0.839	0.2531
7	0.138	0.203	0.0803	0.341	0.1274	0.656	0.859	0.2591
8	0.123	0.326	0.1226	0.449	0.1611	0.542	0.862	0.2598
9	0.136	0.377	0.1389	0.513	0.1798	0.481	0.858	0.2589
10	0.157	0.434	0.1565	0.591	0.2017	0.408	0.842	0.2553
11	0.209	0.507	0.1781	0.716	0.2345	0.281	0.788	0.2425
12	0.211	0.530	0.1847	0.741	0.2407	0.256	0.786	0.2420
13	0.213	0.614	0.2078	0.827	0.2617	0.170	0.784	0.2417
14	0.243	0.665	0.2214	0.980	0.2806	0.089	0.754	0.2345
15	0.131	0.818	0.2596	0.949	0.2898	0.490	0.867	0.2608
16	0.080	0.880	0.2742	0.960	0.2923	0.038	0.918	0.2723
17	0.104	0.849	0.2669	0.953	0.2907	0.046	0.895	0.2672
18	0.094	0.863	0.2702	0.957	0.2915	0.042	0.905	0.2693
19	0.093	0.879	0.2739	0.972	0.2949	0.027	0.906	0.2695

To determine series of consecutive points which are used to calculate a regression line, the following significance test is used.

Table IX. Determination of Points Belonging to a Specific Phase of the Cell Cycle. Experiment II. Determination of Transition From the M + G_1 Plane to S.

Hour	Cumulated Mean ($\bar{X}$)	Hourly Mean ($\bar{X}_f$)	S.E. $\bar{X}$ (Cumulated)
2	1.1385	1.1385	0.0953
2-3	1.2026	1.2882	0.0806
2-4	1.5594	2.1839*	0.1760

*Significant at 2 S.E. $\bar{X}$.

Applying the test, we compare $\bar{X} \pm 2$ (S.E. $\bar{X}$) with $\bar{X}_f$ from the next hour. For example, 1.2882 is within the range of $1.1385 \pm 2(0.0953)$, and therefore cells harvested at hours 2 and 3 are representative of the same phase. However, 2.1839 falls without the range of $1.2026 \pm 2(0.0806)$, and we reject the sample at hour 4 as belonging to the next phase of the cycle.

The series of points which belong to the single-cell kinetics regression during the first S-period was determined by applying a 90% confidence interval to the population kinetics regression. (See Fig. 7.) Points excluded from the interval (triangles), but still falling in S were designated those belonging to the single-cell regression. In turn, a 90% confidence interval plotted about these points is found to exclude population regression points (circles).

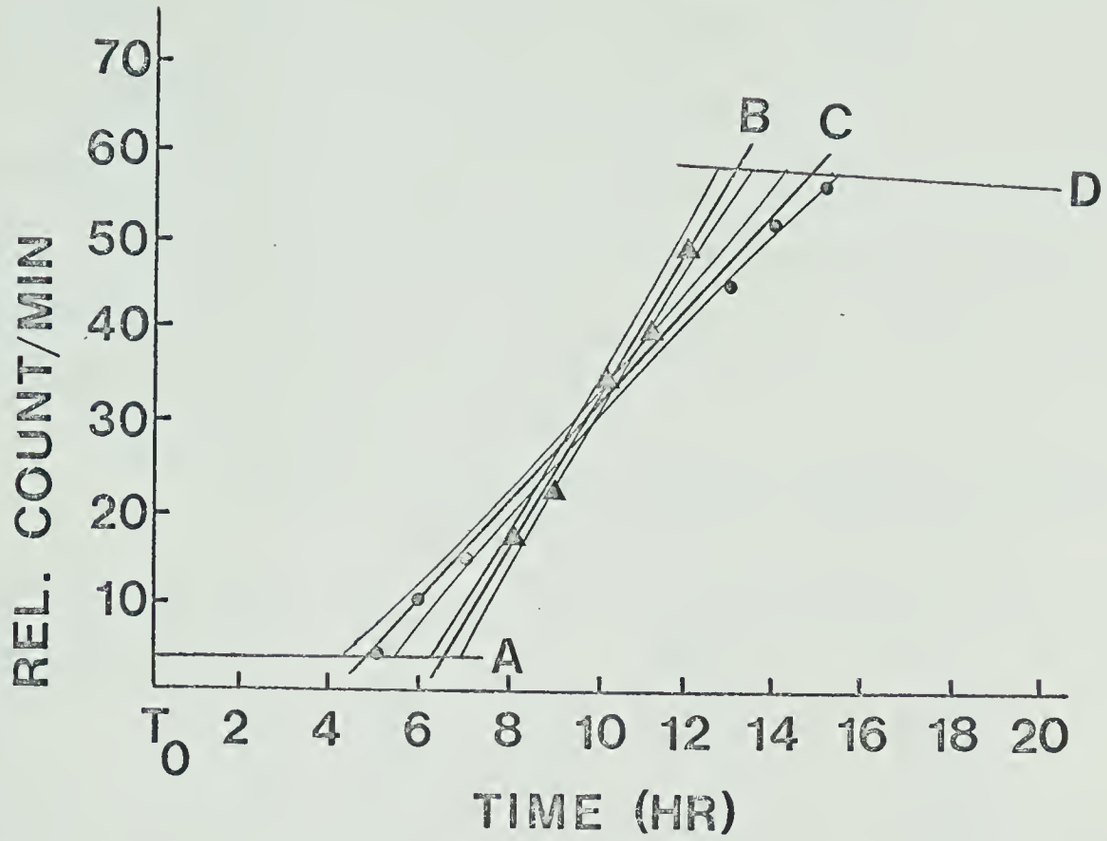

Fig. 7. Determination of Points Which Apply to Population and Single-Cell Kinetics.

A M + G_1 regression.

B Single-cell population regression with 90% confidence interval.

C Population kinetics regression with 90% confidence interval.

D G_2 + M + G_1 regression.

Test for Radiation-Induced Lag

To test the possibility that the higher radiation level used in the scintillation counting method lengthened the cell cycle, the averaged cell cycle durations derived by both methods were examined using Student's t-test. A theoretical slope, β_0, was calculated from replicate scintillation counting experiments and compared with the pooled slope (b) determined from collection function data

(Log (1 + N(M)) and Log (1 + N(M*))) from the autoradiography experiments. (Slope of collection function = $\frac{0.301}{T}$.) It is possible to pool slopes of the collection functions if they can be shown to be homogeneous using the F-test. (Steel and Torrie, 1960, pp 173-174.) Table X. includes pertinent data from the autoradiographic cell cycle determinations to show that a single slope fits the four sets of data from the collection functions.

Table X. Analysis of Variance Table for Testing Differences Between Four Slopes

Source of Variation	df	Σx^2	Σy^2	Σxy	$\frac{(\Sigma xy)^2}{\Sigma x^2}$	df	Residual SS	df
Sple. 1	15	340	0.1217	6.3234	0.1142	1	0.0175	14
Sple. 2	15	340	0.1292	6.5135	0.1247	1	0.0045	14
Sple. 3	15	340	0.1363	6.8038	0.1361	1	0.0002	14
Sple. 4	15	340	0.1154	6.1967	0.1129	1	0.0025	14
Totals					0.4879	4	0.0147	56
Pooled Regression	60	1360	0.5026	25.7464	0.4874	1		
Reduction in SS					0.0005	3		

$$F^{3}_{56} = \frac{\frac{0.0005}{3}}{\frac{0.0147}{56}} = 0.642, \text{ which is not significant.}$$

This leads to the conclusion that the four slopes are homogeneous and can be compared with β_0.

$$\beta_0 = \frac{0.301}{T} = \frac{0.301}{16.05} = 0.01875.$$

$$b \text{ (pooled slope)} = \frac{\Sigma xy}{\Sigma x^2} = 0.0189 \pm 0.003834.$$

$$S^2_{y.x} \text{ (S.E. of estimate)} = \frac{\Sigma y^2 - \frac{(\Sigma xy)^2}{\Sigma x^2}}{n - 2}$$, where n is the number of samples.

$$= 0.0002.$$

$$t = \frac{b - \beta_0}{\sqrt{S^2_{y.x}/\Sigma x^2}} = \frac{0.0189 - 0.01875}{\sqrt{0.0002/1360}} = 0.03912.$$

This value is not significant ($P > 0.5$), and therefore the

radiation level did not induce delay in cell traverse.

Autoradiographic Cell Cycle Determination

The equations used to determine the various phases of the cycle are derived by Puck and Steffen (1963). Data presented are from Experiment I.

Total Cycle Time (T)

This factor is related to the collection function of mitotic cells as they are arrested by colchicine.

Slope of collection function = 0.301/T.

Therefore, T = 0.301/Slope.

For Log (1 + N(M)), T = 0.301/ 0.0195 = 15.38 hr.

For Log (1 + N(M*)), T = 0.301/ 0.0183 = 16.45 hr.

T av. = 15.91 hr.

Mitosis (M)

Duration of mitosis is determined from the doubling function, $2^{\frac{T(M)}{T}}$. At T_0, the fraction of the cell population in mitosis, N(M), is equal to $2^{\frac{T(M)}{T}} - 1$. Solving the equation,

$$0.039 = 2^{\frac{T(M)}{T}} - 1,$$

$$T(M) = 0.88 \text{ hr.}$$

Duration of G_2

The difference in total and labeled mitotic cells reflects the fraction of cells originally in G_2 ($N(G_2)$). This difference, averaged over the period of 15-18 hr, is found to be 0.1017. Corrected for cell multiplication as the cycle progresses, this becomes,

$$0.1017 \times 2^{0.0551} = 0.1056.$$

The following equation is used to determine $T(G_2)$.

$$N(G_2) - N(M) = 2^{\frac{T(M)}{T}} \left(2^{\frac{T(M + G_2) - T(M)}{T}} - 1 \right)$$

When solved, this equation gives $T(G_2) = 1.1$ hr. $T(G_2)$ may also be determined directly from the plot of labeled and total mitoses vs. time (see Fig. 4. (a.) and (c.)). From the above value of $T(G_2)$, the value of $N(G_2)$ is recalculated.

$$N(G_2) = \frac{T(G_2)}{T} = 0.72$$

The averaged value of $T(G_2)$ is used to determine k, a constant required in the determination of S.

Duration of S Phase

The length of S period is determined from the intercept of a plot of the accumulation function, Log $(1 + N(L)/k)$ vs. time. $N(L)$ represents the fraction of the cell population which is labeled, and k is the constant, $2^{\frac{T(G_2)}{T}}$. The intercept of this function is equal to $0.301\ T(S)/T$ (see Figs. 4. (b.) and (d.)). For Experiment I.,

$$0.1795 = 0.301\ T(S)/T,$$

from which $T(S) = 9.49$ hr.

Duration of G_1

The final step is the determination of $T(G_1)$. The graph of the accumulation function gives this information (see Figs. 4. (b.) and (d.)). The asymptotic convergence of two regression lines of the two phases of the curve is said to denote the duration of $M + G_1$. $T(G_1)$ is obtained by subtraction, and is found to be 2.12 hr.